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TITLE OF THE INVENTION (280 characters max)

PEPTIDE CONJUGATES FOR THE STABILIZATION OF MEMBRANE PROTEINS AND INTERACTIONS WITH BIOLOGICAL MEMBRANES

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PROVISIONAL APPLICATION FILING ONLY

Title

Peptide conjugates for the stabilization of membrane proteins and interactions with biological membranes

Field of the Invention

This invention generally relates to compounds that have utility as detergents. In particular, the present invention relates to a novel class of peptide-based chemical compounds that interact with proteins, lipids and other molecules. The compounds may be used for the stabilization and crystallization of proteins and membrane proteins, in particular. The compounds are also useful for modifying the properties of lipid bilayer membranes, and have potential uses as cytolytic agents, as molecules that can facilitate the transport of polar molecules across biological membranes, and as emulsifiers and surfactants.

Background of the Invention

Membrane proteins are critical components of all biological membranes, and can function as enzymes, receptors, channels and pumps. They are also very common in biological systems, and constitute 20-40% of all proteins found in the bacteria, archaea and eukaryotes (Wallin and von Heijne, *Protein Sci*, **7,** 1029-38 (1998), Boyd, et al., *Protein Sci*, **7,** 201-5 (1998), Gerstein, *Proteins*, **33,** 518-34 (1998), Jones, *FEBS Lett*, **423,** 281-5 (1998), Arkin, et al., *Proteins*, **28,** 465-6 (1997)). Many clinically useful drugs, including the widely prescribed drugs, Prozac and Prilosec, interact with human membrane proteins. However, despite the abundance and importance of membrane proteins, this class of molecules is still only poorly understood at a structural level, mainly because of difficulties in growing crystals of membrane proteins suitable for analysis by x-ray crystallography (Garavito, et al., *J Bioenerg Biomembr*, **28,** 13-27 (1996), Ostermeier and Michel, *Curr Opin Struct Biol*, **7,** 697-701 (1997), Garavito, Curr Opin Biotechnol 9, 344-349 (1998)).

In order to understand the mechanism of action of a particular membrane protein, it is essential to know the three-dimensional structure of the molecule to a resolution that

reveals its atomic structure. This is typically taken to be better than 0.3 nm resolution, and nearly all of the membrane protein structures that are known to this resolution have been determined by the technique of x-ray crystallography (Branden and Tooze, *Introduction to Protein Structure*, Garland Publishing Inc., New York (1998)). If the protein in question is medically important, knowledge of the 3-dimensional structure of the protein is a prerequisite for the development of new therapeutics using structure-based rational drug design methodologies (Wanke and DuBose, *Pharm Pract Manag Q*, **18**, 13-22 (1998)). The techniques used in the study of membrane protein crystals are very similar to those used for crystals of soluble proteins, and the main barrier to advancement in this field is the generation of diffraction-quality crystals.

The techniques used for the crystallization of membrane proteins are generally similar to the techniques used for the crystallization of soluble proteins. Typically, a purified, concentrated solution of protein is brought to the limit of its solubility over the course of days or weeks, resulting in either the formation of a protein precipitate or of protein crystals. Because precipitates are more often observed than crystals, numerous conditions are tested in these trials. The number of trials can vary in number from a few dozen to several thousand in attempts to find conditions resulting in crystal formation. The tested conditions can differ in pH, nature of added salts, concentration of the added salts, nature of the precipitant, concentration of the precipitant, temperature, and other factors (A. McPherson, in "Crystallization of Biological Macromolecules", Cold Spring Harbour Press (1998)). In some instances, conditions producing suitable crystals for analysis by x-ray diffraction are not discovered even after extensive screening.

If the protein under consideration is an intrinsic membrane protein, the protein sample used in the crystallization trials is first purified and stabilized in a specific detergent in order to preserve the native conformation of the protein in the absence of a lipid bilayer (H. Michel, *Trends Biochem. Sci.* **8**, 56-59 (1983), W. Kuhlbrandt, *Quart. Rev. Biophysics* **21**, 429-477 (1988)). In most instances, a number of different detergents are tested for their ability to stabilize a particular membrane protein, and for their effect in the

crystallization trials. Examples of detergents suitable for these purposes includ the alkyl gylcoside detergents such as octyl β-D-glucopyranoside (OG) and dodecyl β -D - maltopyranoside (DDM) (Baron and Thompson, *Biochim. Biophys. Acta* **382**, 276-285 (1975), Rosevear et al., *Biochemistry* **19**, 4108-4115 (1980)), the polyoxyethylene alkyl ether detergents such as pentaethylene glycol monooctyl ether (C8E5) and octoethylene glycol monododecyl ether (C12E8) (Garavito and Rosenbusch, Meth. Enzymol. **125**, 309-328 (1886), Victoria and Mahan, Biochim Biophys Acta **644**, 226-232 (1981)), and the detergents described in U.S. Patent No. 5,674,987, which are prepared from the reaction of a cycloalkyl aliphatic alcohol and a saccharide. Detergent-solubilized membrane proteins exist as protein-detergent complexes (PDC) in which a ring of detergent molecules covers the surface of the protein that is normally embedded in the lipid bilayer. The hydrophobic portions of the detergent amphiphiles interact with the protein surfaces normally in contact with the lipid acyl chains, and thus mimic the normal lipid environment at the surface of the membrane protein. This micelle-like ring of detergent molecules surrounding the membrane protein is very dynamic and mobile, such that the surface properties of the PDC is in general poorly suited to the formation of well-ordered crystals (Crystallization of Membrane Proteins, H. Michel ed. CRC Press, Boca Raton, FL (1991)). This is unfavorable effect is lessened in cases where the protein has large extramembranous domains, or with detergents that have small micellar volumes.

A number of techniques have been developed to address this difficulty in attempts to achieve membrane protein crystallization. For example, the formation of a complex with an antibody fragment has been used to increase the polar surface area of the *Paracoccus denitrificans* cytochrome oxidase, resulting in well-diffracting crystals (Ostermeier et al., Nat Struct Biol, 2, 842-6 (1995), Ostermeier et al., Proc Natl Acad Sci USA, 94, 10547-53 (1997)). Fusion proteins of the membrane protein lactose permease with soluble carrier domains have been made in attempts to achieve a similar result (Privé et al., Acta Cryst **D50**, 375-379 (1994), Privé and Kaback, J Bioenerg Biomembr **28**, 29-34 (1996)). Bacteriorhodopsin has been crystallized from cubic lipid phases (Landau and

Rosenbusch, Proc Natl Acad Sci U S A, 93, 14532-5 (1996)) in a method that does not rely on detergents at all. However, the general applicability of this method to proteins other than bacteriorhodopsin remains unproven. A strategy to reduce the volume and dynamics of the detergent surface of the PDC has been proposed by Schafmeister et al. (Science, **262**, 734-8 (1993)). In this approach, amphipathic peptides have been used in the place of traditional detergents such as octyl glucoside. The peptides were designed such that the peptide would form an α-helix with one hydrophilic face and one hydrophobic face. The intention was that the hydrophobic surface was of the peptide would associate with the transmembrane surface of a membrane protein. Although the peptide used in this study could maintain some membrane proteins in a solubilized state for a few days, the proteins were not sufficiently stabilized for the purposes of crystallization. Because of their limited effectiveness as detergents, these peptides have not found general utility as tools for the study of membane proteins.

In the traditional detergents consisting of a polar head group and a linear alkyl tail, the length of the hydrocarbon moiety is an important factor in determining the ability of the detergent to preserve the native conformation of a solubilized membrane protein. Within the framework of a common head group, longer chain length detergents are generally more stabilizing towards membrane proteins, and are considered to be more "gentle". The presumed mechanism for stabilization is that the longer chains are deemed to be more effective at masking the hydrophobic transmembrane surface of the membrane protein than the short chain detergents and are thus better mimics of the native membrane environment. However, longer chain detergents occupy a larger volume of the belt region of the PDC, a feature that is expected to reduce the probability of crystallization of the complex (Michel, 73-87 in "Crystallization of Membrane Proteins", H. Michel, ed., CRC Press. Boca Raton, FL (1991)). Another factor affecting the choice of a particular detergent is the solubility of the detergent in water or buffer solutions. As the alkyl chain length increases in a series of detergents with a common head group, the overall solubility of the detergent decreases, eventually to levels making the detergent impractical for most

uses. Thus, octyl glucoside is soluble to levels greater than 20% in water, while decyl glucoside is soluble to only 0.1% in similar conditions, and dod cyl glucoside is soluble only to 0.008% (Anatrace Inc., Maumee OH 1999-2000 Catalogue). With a larger head group such as maltoside, the solubility of the long chain detergents increases, but solubility is still reduced to impractical levels with hexadecyl chain lengths or longer. Thus, within a series of traditional detergents, there is confict in the preferred length of the alkyl chain length. Long chains favor protein stability, and short chains are optimal for crystallization and detergent solubility. Since protein stability is a prime concern for crystallization trials, many membrane protein crystallization trials are carried out under sub-optimal conditions.

There is a need, thus, for a non-denaturing detergent which effectively mimics the membrane's lipid bilayer, is capable of solubilizing membrane proteins in such a way that the three-dimensional conformation necessary for crystallization is retained, and has features to enhance the probablility of crystallization of membrane proteins.

Summary of the Invention

Accordingly, in one aspect, the present invention provides an amphipathic peptide conjugate having detergent properties and having a hydrophobic face and a hydrophilic face, said peptide moiety of the conjugate comprising a first end and a second end, wherein said first end is covalently linked to a first aliphatic hydrocarbon moiety and said second end is covalently linked to a second aliphatic hydrocarbon moiety, said aliphatic moieties linked such that they associate with the peptide moiety of the conjugate.

Preferably the peptide conjugate is a lipopeptide detergent.

Generally, a purified protein in a known detergent is subjected to a process whereby the known detergent is exchanged for the novel detergent of the present invention. The protein in the novel detergent may then be subjected to conditions that promote crystallization to occur.

Brief Description of the Drawings

The present invention is described in further detail herein by reference to the following drawings in which:

Figure 1A is a schematic representation of a single lipopeptide detergent (LPD) molecule in accordance with the present invention;

Figure 1B is a schematic representation of a cylindrical assembly of several lipopeptides in which the aliphatic hydrocarbon tails are clustered in the core of the assembly;

Figure 1C is a schematic representation of a membrane protein solubilized by a traditional detergent (prior art);

Figure 1D is a schematic representation of a membrane protein solubilized by a lipopeptide detergent in accordance with the present invention;

Figure 2 is a graph showing the effectiveness of 5 lipopeptide detergents in maintaining the membrane protein bacteriorhodopsin in a soluble, stable state in the absence of a phospholipid membrane; and

Figure 3 is a scatter plot demonstrating that the lipopeptide detergents interact with phospholipid membranes, dissolving them into micelles.

Detailed Description of the Invention

Detailed Description of the Drawings

Figure 1 is a schematic representation of the lipopeptide detergents. Figure 1A shows a single LPD molecule with the α-helical peptide represented in a Cα tracing with grey lines, and the aliphatic acyl chains of two fatty acids coupled to side chains at either end of the peptide shown with black lines. This representation is the presumed conformation of the monomer within the assembly shown in Figure 1B. Figure 1B shows the presumed assembly of the peptides into a cylindrical assembly. The fatty acyl chains cluster in the core of the assembly, near the central axis of the cylinder. Figure 1C shows a schematic representation of a membrane protein solubilized by a traditional detergent. This is

included to show the contrast between the present invention and the prior art. Figure 1D shows a similar protein solubilized by a lipopeptide detergent.

Figures 2 and 3 are discussed in detail later in the description.

The present invention provides novel lipopeptide detergents comprising an α-helical peptide scaffold having aliphatic hydrocarbon tails covalently linked to opposite ends of the peptide scaffold.

The peptide scaffold is not particularly limited with respect to its amino acid sequence. However, the amino acid sequence is selected so as to permit formation of the peptide scaffold into an amphipathic α-helical conformation. Generally, the peptide will comprise a mixture of hydrophobic and hydrophilic regions. Hydrophobic regions will include, but are not limited to, neutral or hydrophobic amino acids such as alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophane or amino acids that do not occur in nature. Preferably, the hydrophobic regions are alanine-rich to favor the formation of an α-helical conformation (Chakrabartty et al., Protein Sci, 3, 843-52 (1994). The hydrophilic regions will include, but are not limited to, amino acids which are primarily hydrophilic in nature such as glutamate, lysine, glutamine, aspartate, asparaginine, histidine, serine, tyrosine, threonine or amino acids that do not occur in nature. Preferably, the hydrophilic regions promote helix formation through the formation of (i,i+4) salt bridges (Marqusee and Baldwin, Proc Natl Acad Sci U S A, 84, 8898-902 (1987)). The hydrophilic regions of the peptide align on the face of the helix that will interact with bulk aqueous phase when in a lipopeptide assembly as shown in Figure 1B. The neutral or hydrophobic face will include two residues for covalent coupling of the aliphatic moieties in the peptide conjugate. These residues will be near the termini of the peptide, at positions where they are aligned with the hydrophobic face of the peptide. The two residues can be lysine, ornithine, cysteine, glutamate or aspartate residues, but are not limited to these amino acids. Preferably, the two residues are ornithines.

The number of amino acids in the peptide scaffold is variable, and will generally be selected such that the length of the peptide scaffold when in an α-helical conformation will approximate the width of a natural membrane phospholipid bilayer, i.e. between 3.0 - 4.5 nm. Accordingly, the number of amino acids in the peptide scaffold will range from about 15 to 35 amino acids. Preferably, the number of amino acids in the scaffold will be about 20 - 30. More preferably, the peptide scaffold will include about 25 amino acids, or a number of amino acids which when in an α-helical conformation measure a length of about 3.7 nm.

The terminal amino acids of the peptide scaffold are also selected to promote α-helix formation, and may be naturally occurring amino acids or modified forms thereof. Modifications commonly made to terminal amino acids in peptides include the addition of groups conventionally used in the art of peptide chemistry which will not adversely affect the function of the lipopeptide. For example, suitable N-terminal blocking groups can be introduced by alkylation or acylation of the N-terminus. Examples of suitable N-terminal blocking groups include C_1-C_5 branched or unbranched alkyl groups, acyl groups such as formyl and acetyl groups, as well as substituted forms thereof, such as the acetamidomethyl (Acm) group. Desamino analogs of amino acids are also useful N-terminal blocking groups, and can either be coupled to the N-terminus of the peptide or used in place of the N-terminal residue. Suitable C-terminal blocking groups, in which the carboxyl group of the C-terminus is either incorporated or not, include esters, ketones or amides. Ester or ketone-forming alkyl groups, particularly lower alkyl groups such as methyl, ethyl and propyl, and amide-forming amino groups such as primary amines (-NH_2), and mono- and di-alkylamino groups such as methylamino, ethylamino, dimethylamino, diethylamino, methylethylamino and the like are examples of C-terminal blocking groups. Descarboxylated amino acid analogues such as agmatine are also useful C-terminal blocking groups and can be either coupled to the peptide's C-terminal residue or used in place of it. Further, it will be appreciated that the free amino and carboxyl groups at the termini can be removed altogether from the peptide to yield desamino and descarboxylated forms thereof without affect on peptide function.

Preferred examples of such modifications include N-terminal acetylation and C-terminal amidation which are known to promote α-helix formation (Doig et al., Biochemistry, 33, 3396-403 (1994)).

Internal amino acids of the peptide may also be modified by derivatization provided that this modification does not affect the function of the lipopeptide, and does not interfere with its α-helical conformation. Such derivatizations can be made to the side chains of the amino acids. For example, the side chains can derivatized by incorporation of blocking groups as described above.

The peptide conjugate may be readily prepared by standard, well-established solid-phase peptide synthesis (SPPS) as described by Stewart et al. in Solid Phase Peptide Synthesis, 2nd Edition, 1984, Pierce Chemical Company, Rockford, Illinois; and as described by Bodanszky and Bodanszky in The Practice of Peptide Synthesis, 1984, Springer-Verlag, New York. Other synthetic protocols, including biological or solution phase methods, can also be used. For the SPPS method, a suitably protected amino acid residue is first attached through its carboxyl group to a derivatized, insoluble polymeric support, such as cross-linked polystyrene or polyamide resin. "Suitably protected" refers to the presence of protecting groups on both the α-amino group of the amino acid, and on any side chain functional groups. Side chain protecting groups are generally stable to the solvents, reagents and reaction conditions used throughout the synthesis, and are removable under conditions which will not affect the final peptide product. Stepwise synthesis of the oligopeptide is carried out by the removal of the N-protecting group from the initial amino acid, and coupling thereto of the carboxyl end of the next amino acid in the sequence of the desired peptide. This amino acid is also suitably protected. The carboxyl of the incoming amino acid can be activated to react with the N-terminus of the support-bound amino acid by formation into a reactive group such as formation into a carbodiimide, a symmetric acid anhydride or an "active ester" group such as hydroxybenzotriazole or pentafluorophenyl esters.

Examples of solid phase peptide synthesis methods include the BOC method which

utilizes tert-butyloxycarbonylas the α-amino protecting group, and the FMOC method which utilizes 9-fluorenylmethyloxycarbonylto protect the α-amino of the amino acid residues, both methods of which are well-known by those of skill in the art.

The aliphatic moieties can be coupled to the resin-coupled peptide by selectively deblocking amino acid side chain protecting groups, followed by reaction with an appropriate aliphatic derivative. Aliphatic derivatives suitable for this purpose include, but are not limited to, saturated fatty acids, unsaturated fatty acids, branched fatty acids, cyclic alkyl acids, aromatic alkyl acids, alkyl amines, alkyl maleimides, alkyl acid chlorides, and alkyl anhydrides. Several strategies can be used to couple the aliphatic derivative to the peptide. For example, if the peptide is synthesized with the FMOC method, a BOC group can be used as the protecting group on the δ-amino group of the ornithine monomers identified as sites for aliphatic coupling. Upon completion of the synthesis of the main peptide chain, the ornithine BOC groups can be selectively removed with trifluoroacetic acid, generating free primary amino functionalities at these positions. Reaction with an aliphatic derivative such as a fatty acid can be used to form a peptide linkage with each of the two ornithine side chains. Examples of suitable saturated fatty acids include octanoic acid, nonanoic acid, decanoic acid, undecanoic acid, dodecanoic acid, tridecanoic acid, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, hexadecanoic acid, heptadecanoic acid, heptadecanoic acid, octadecanoic acid, nondecanoic acid, eicosanoic acid, heneicosaoic acid, docosanoic acid, tricosanoic acid, tetracosanoic acid, pentacosanoic acid, and hexacosanoic acid. Following the coupling of the aliphatic groups, the remaining amino acid side chains can be deblocked under appropriate conditions, such as with hydrofluoric acid or trifluoromethanesulfonicacid.

Incorporation of N- and/or C- blocking groups can also be achieved using protocols conventional to solid phase peptide synthesis methods. For incorporation of C-terminal blocking groups, for example, synthesis of the desired peptide is typically performed using, as solid phase, a supporting resin that has been chemically modified so that cleavage from the resin results in a peptide having the desired C-terminal blocking group. To provide

peptides in which th C-t rminus bears a primary amino blocking group, for instance, synthesis is performed using a p-methylbenzhydrylamine(MBHA) resin so that, when peptide synthesis is completed, treatment with hydrofluoric acid or trifluoromethanesulfonic acid (TFMSA) releases the desired C-terminally amidated peptide. Similarly, incorporation of an N-methylamine blocking group at the C-terminus is achieved using N-methylaminoethyl-derivatized DVB resin, which upon HF treatment releases a peptide bearing an N-methylamidated C-terminus. Blockage of the C-terminus by esterification can also be achieved using conventional procedures. This entails use of resin/blocking group combination that permits release of side-chain protected peptide from the resin, to allow for subsequent reaction with the desired alcohol, to form the ester function. FMOC protecting groups, in combination with DVB resin derivatized with methoxyalkoxybenzylalcohol or equivalent linker, can be used for this purpose, with cleavage from the support being effected by TFA in dicholoromethane. Esterification of the suitably activated carboxyl function e.g. with DCC, can then proceed by addition of the desired alcohol, followed by deprotection and isolation of the esterified peptide product.

Incorporation of N-terminal blocking groups can be achieved while the synthesized peptide is still attached to the resin, for instance by treatment with a suitable anhydride and nitrile. To incorporate an acetyl blocking group at the N-terminus, for instance, the resin-coupled peptide can be treated with 20% acetic anhydride in acetonitrile. The N-blocked peptide product can then be cleaved from the resin, deprotected and subsequently isolated.

To ensure that the peptide obtained from either chemical or biological synthetic techniques is the desired peptide, analysis of the peptide composition should be conducted. Such amino acid composition analysis may be conducted using high resolution mass spectrometry (MS) to determine the molecular weight of the peptide. Alternatively, or additionally, the amino acid content of the peptide can be confirmed by hydrolyzing the peptide in aqueous acid, and separating, identifying and quantifying the components of the mixture using HPLC, or an amino acid analyzer. Protein sequenators, which sequentially degrade the peptide and identify the amino acids in order, may also be used to determine

definitely the sequence of the peptide.

Having obtained the desired peptide conjugate, purification to remove contaminants is generally then conducted. Any one of a number of conventional purification procedures may be used to attain the required level of purity including, for example, ion-exchange and gel filtration chromatography or reversed-phase high-pressure liquid chromatography (HPLC) using an alkylated silica column such as C_4-, C_8- or C_{18}- silica. A gradient mobile phase of increasing organic content is generally used to achieve purification, for example, acetonitrile in an aqueous buffer, usually containing a small amount of trifluoroacetic acid. Because the overall hydrophobicity of the peptide conjugates increases with larger aliphatic moieties, C_4-silica is the preferred chromatographic resin for these compounds.

Aliphatic hydrocarbon moieties are linked in a covalent manner to both the N- and C-termini of the scaffold peptide or to sites near each of these termini such that they associate with the hydrophobic region of the peptide scaffold. In one embodiment of the present invention, the aliphatic hydrocarbon tails are linked to ornithine residues located adjacent to N- and C- terminal alanine residues of the scaffold peptide. The δ-amino groups of the ornithines are coupled to the carboxyl groups of tetracosanoic acid via peptide linkages. Ornithines are used in place of the more common lysine residues as sites for the hydrocarbon tail linkage since they have fewer methylene groups between the main chain peptide atoms and the side chain amine, and may position the hydrocarbon chains more precisely in association with the hydrophobic region of the peptide.

The lipopeptide detergent is advantageous over "traditional" detergents due to its ability to self-associate into a cylinder of defined dimensions. The cylinders are made up of colinear α-helices and themselves associate into a cylindrical assembly, as shown in Fig. 1B, in which the hydrophilic surfaces of the individual helices are exposed to the bulk aqueous phase and the hydrocarbon tails are packed in the core of the assembly effectively mimicking the chains in a membrane phospholipid bilayer. Fig. 1D illustrates how a membrane protein can be accommodated in the core of a lipopeptide assembly with the aliphatic hydrocarbon tails forming a cylindrical layer against the protein, again

better mimicking biological membrane conformation, allowing for preservation of the biological activity of solubilized membrane proteins.

The described lipopeptide detergents with two coupled aliphatic moieties ranging from ten to twentyfour carbon alkyl chains are soluble in water, in contrast to an alkyl chain length maximum of sixteen carbon groups in the traditional detergents. The favorable solubility properties of the long chain lipopeptide detergents make it possible for these detergents to stabilize large hydrophobic surfaces of membrane proteins.

In addition to their stabilizing properties, the present lipopeptide detergents have been designed to favor the crystallization of membrane proteins. They lie close to the surface of the membrane protein, and are thus less obtrusive to the formation of a crystal lattice. Also, they present a rigid outer surface of α-helices. These are features that favor membrane protein crystallization (Schafmeister et al., Science, 262. 734-8 (1993) Michel, Crystallization of membrane proteins, 73-87 (1991)).

The lipopeptide detergents of the present invention may be used to crystallize membrane proteins. Generally, the method comprises solubilizing the membrane protein with a detergent, and then exposing the solubilized membrane to conditions which promote crystallization to occur.

The lipopeptide detergents are also membrane-active compounds, and can insert into phospholipid bilayers. At sufficiently high concentrations, they can disrupt the bilayers and form mixed lipid/lipopeptide micelles.

The lipopeptide detergents of the present invention have the activities of traditional detergents and hence they may be used to modulate and disrupt biological membranes, and therefore to transport polar molecules across membranes, including ions. As surface active agents or emulsifiers, they may be used in protein and/or lipid emulsions. They may also be used as cytolytic agents.

EXAMPLES

Embodiments of the present invention are described in further detail by reference to the following specific examples, which are not to be construed as limiting the appended claims.

Example 1: Synthesis of LPD-24

The lipopeptide, LPD-24, exemplifies a lipopeptide detergent in accordance with the present invention. The scaffold peptide of LPD-24 has the following chemical structure:

CH3CONH-AOAEAAEKAAKYAAEAAEKAAKAOA-CONH2

wherein A is alanine, O is ornithine, E is glutamate, K is lysine, and Y is tyrosine, CH3CONH- is the acetylated amino terminal group of the peptide, and –CONH2 is the carboxamide end of the peptide chain. A single tyrosine is included to allow spectrophotometric detection of the peptide at 280 nm.

LPD-24 is synthesized on a solid support resin using a combination of tert-butoxycarbonyl (Boc) and 9-Fluorenylmethoxycarbonyl (Fmoc) chemistries (Novabiochem 97/98 Catalogue and Peptide synthesis handbook). The synthesis proceeds from the C-terminus of the peptide to the N-terminus, with all the main chain peptide synthesis couplings based on Fmoc chemistry. The resin tert-butoxycarbonyl-Alanine-methylbenzhydrylamine (Boc-Ala-MBHA) is chosen so as to produce a peptide carboxamide upon cleavage from the resin. The resin is first prepared by removal of the Boc protecting group with 50% trifluoroacetic acid (TFA), generating the free α-amino acid amine of the alanine. Sequential addition of the following 24 amino acids proceeds with the coupling of the appropriate Fmoc-protected amino acid: N-α-Fmoc-L-alanine (Fmoc-Ala), N-α-Fmoc-L-glutamamic acid α-benzyl ester (Fmoc-Glu(Obz)), N-α-Fmoc-N-α-2-chloro-benzyloxycarbonyl-L-lysine (Fmoc-Lys(2ClZ)), N-α-Fmoc-N-α-tertbutoxycarbonyl-L-ornithine (Fmoc-Orn(Boc)), or N-α-Fmoc-O-2-bromo- benzyloxycarbonyl-L-tyrosine (Fmoc-Tyr(2BrZ)) with the coupling reagent O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HATU). Upon completion of the coupling

reaction, the Fmoc protecting group is removed with 20% piperidine in preparation for the next amino acid coupling. Following the addition of the last amino acid and the removal of the Fmoc group, the amine terminus of the chain is acetylated with acetic anhydride. Next, the Boc protecting groups of the ornithine side chains are removed with 50% TFA in preparation for the coupling with the fatty acid. Two equivalents of tetracosanoic acid are coupled to the peptide with HATU. The final step involves the cleavage of the peptide from the resin and the deprotection of the glutamate, lysine, and tyrosine side chains with trifluoromethanesulfonic acid (TFMSA).

The lipopeptide is precipitated in ether, and washed four times in ether. The white pellet is dissolved in water, lyophilized, and redissolved in water. The peptide is purified by gel filtration chromatography in ammonium carbonate buffer, lyophilized, and redissolved in water. The correctness of the synthesis is confirmed by electrospray ionization mass spectrometry (ESI-MS).

Lipopeptide detergents with pairs of aliphatic hydrocarbon tails of length 10, 12, 14, 16, 18, 20, 22, 24, and 28 carbons (LPD-10 to LPD-28) have been designed and synthesized. The LPDs with chain lengths up to 24 carbons are soluble in water to over 50 mg/ml. LPD-28 is poorly soluble in water. Computer-assisted molecular modelling suggests that the longer chains (C16 and greater) can cross past each other and that each may span the entire length of the helix. Every batch of peptide is analyzed by ESI-MS to confirm the synthesis. The uncoupled peptide is referred to as LPD-0, and does not have detergent properties.

Example 2: Effectiveness of Lipopeptides in stabililizing solubilized membrane proteins

Bacteriorhodopsin was purified from purple membranes (isolated from *Halobacterium halobium*, a generous gift from J.Lanyi, UC Riverside). 4 mL of purple membranes were diluted with 10 ml 1% Triton X100, 10 mM NaH2PO4 pH 5.7, and mixed for 12 hours at 30°C in the dark. The solution was then centrifuged for 15 minutes at 55,000 r.p.m in a Beckman NVT 90 centrifuge rotor to remove any insoluble material. The supernatant was

added to a diethylaminoethyl (DEAE) sepharose ion exchange column (10 ml column volume) previous equilibrated with 1% Triton X100, 10 mM NaH2PO4 pH 5.7. The column was then washed with 25 mL 34 mM OG, 10 mM NaH2PO4 pH 5.7, followed by elution with a buffer containing 34 mM OG, 500 mM NaH2PO4 pH 5.7. OG is a standard detergent for the purification and crystallization of bacteriorhodopsin (G.F. Schertler et al., J. Mol. Biol **234**, 156-164 (1993)). LPD (lipopeptide) detergents (LPD-12, LPD-14, LPD-16, LPD-18 and LPD-20) were added to the protein solutions at a final concentration of 3.25 mM, and the OG was removed by dialysis in the dark through membranes with molecular weight cutoffs of 1 kDa. The LPD detergents were fully retained in the protein solution, while the OG was removed from the protein solution to levels of less than 0.5 mM after 2 days. The samples were stored in the dark at room temperature, and at 1 day, 4 days, 12 days, 18 days and 25 days storage, the samples were centrifuged at 100,000 X g for 45 minutes and the absorbance at 540 nm of the supernatants were measured. Solubilized, properly folded, native bacteriorhodopsin remains in the supernatant and has an absorbance maximum at 540 nm. All LPDs with chain lengths of 12 amino acids and longer were much more stabilizing to the membrane protein bacteriorhodopsin than octyl glucoside (OG) as illustrated in Fig. 2.

Example 3: Interaction of lipopeptide detergents with phospholipid membranes

Phosphatidyl choline vesicles were prepared by extrusion through 0.1 nm pore membranes (Avestin, Ottawa), at 1 mM concentration, and diluted to 0.1 mM phospholipid. Dodecyl maltoside (DDM) or lipopeptide detergent were added to the indicated concentrations, and the hydrodynamic radius of the suspension was measured on a DynaPro-800 dynamic light scattering device.

The results of this experiment are illustrated in Fig. 3. This scatter plot demonstrates that the lipopeptide detergents interact with phospholipid membrane vesicles, dissolving them into micelles. The control C-0 peptide had no effect on these vesicles.

The disclosure of all of th literature references and any patents referred to herein are incorporated herein by reference.

While the invention has been described with particular reference to certain embodiments thereof, it will be understood that those of ordinary skill in the art within the scope and spirit of the following claims may make changes and modifications.

In the claims, the word "comprising" means "including the following elements (in the body), but not excluding others"; the phrase "consisting of" means "excluding more than traces of other than the recited ingredients"; and the phrase "consisting essentially of" means "excluding unspecified ingredients which materially affect the basic characteristics of the composition".

I Claim:

1. An amphipathic peptide conjugat having detergent prop rties and having a hydrophobic face and a hydrophilic face, said peptide moiety of the conjugate comprising a first end and a second end, wherein said first end is covalently linked to a first aliphatic hydrocarbon moiety and said second end is covalently linked to a second aliphatic hydrocarbon moiety, said aliphatic moieties linked such that they associate with the peptide moiety of the conjugate.
2. The peptide conjugate as defined in claim 1, which comprises a lipopeptide detergent.
3. The peptide conjugate as defined in claim 1, wherein said peptide comprises hydrophobic and hydrophilic regions.
4. The peptide conjugate as defined in claim 1, wherein said peptide comprises 15-35 amino acids.
5. The peptide conjugate as defined in claim 4, wherein said peptide comprises about 25 amino acids.
6. The peptide conjugate as defined in claim 5, wherein said peptide has the amino acid sequence, AOAEAAEKAAKYAAEAAEKAAKAOA.
7. The peptide conjugate as defined in claim 1, wherein the length of said peptide is approximately equal to the width of a phospholipid bilayer.
8. The peptide conjugate as defined in claim 7, wherein the length of said peptide is in the range of about 3.5 - 4.0 nm.
9. The peptide conjugate as defined in claim 8, wherein the length of said peptide is about 3.7 nm.
10. The peptide conjugate as defined in claim 1, wherein the termini of said peptide are protected.
11. The peptide conjugate as defined in claim 10, wherein the N-terminus of said peptide is acetylated and the C-terminus of said peptide is amidated.
12. The peptide conjugate as defined in claim 1, wherein said aliphatic hydrocarbon moieties comprise from about 8-24 carbon atoms.

ABSTRACT

The present invention provides a novel class of detergents referred to herein as lipopeptide detergents. Lipopeptide detergents comprise an amphipathic α-helical peptide having a hydrophobic or neutral face and a hydrophilic face. To both ends of this peptide is covalently linked aliphatic hydrocarbon tails, these aliphatic tails being linked thereto such that they associate with the hydrophobic or neutral face of the peptide. Lipopeptide detergents can advantageously be used to solubilize membrane proteins in a manner that permits the subsequent crystallization thereof.

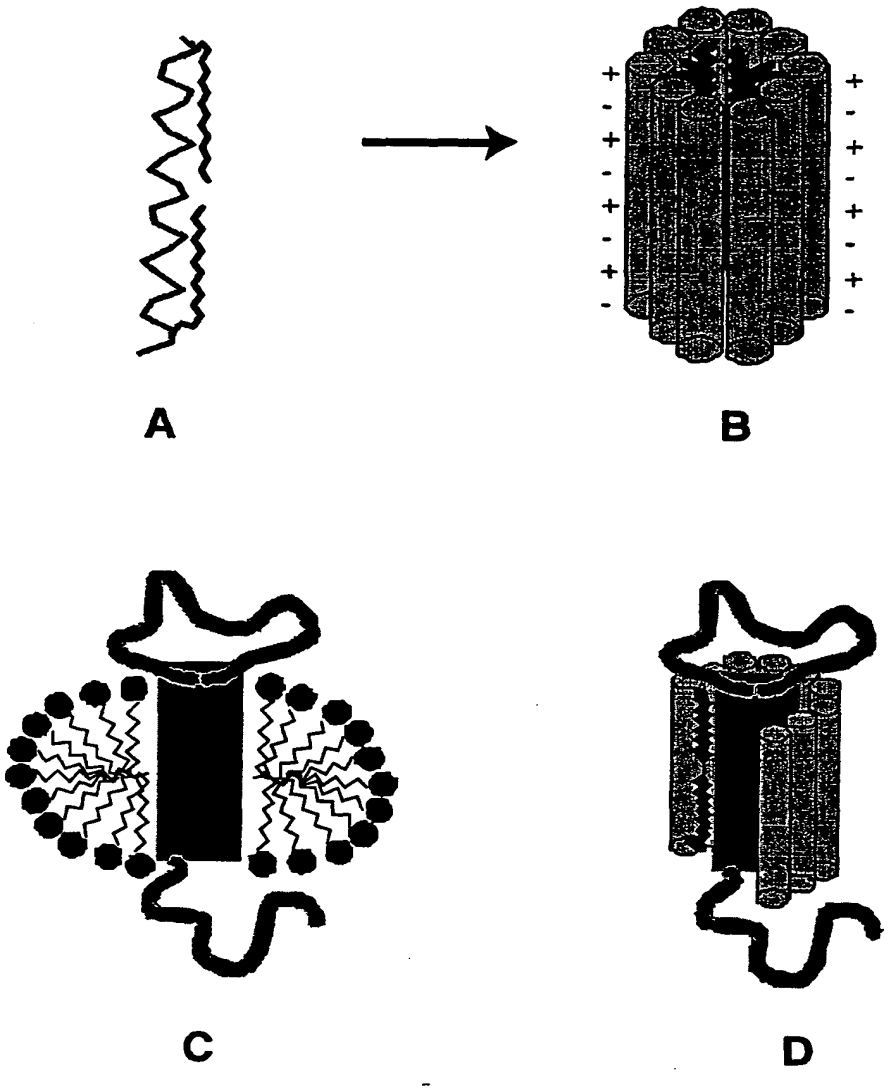

Figure 1

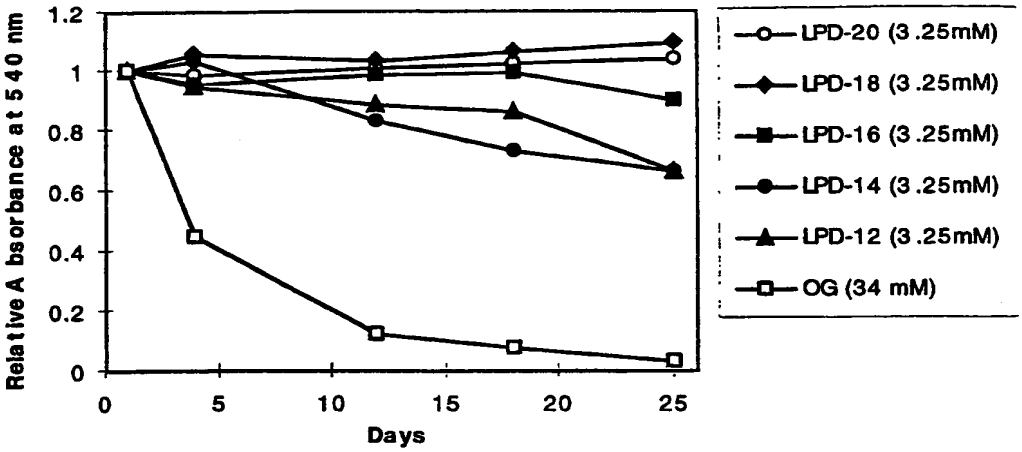
Relative A bsorbance at 540 nm
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
Days
LPD-20 (3.25mM)
LPD-18 (3.25mM)
LPD-16 (3.25mM)
LPD-14 (3.25mM)
LPD-12 (3.25mM)
OG (34 mM)

Figure 2.

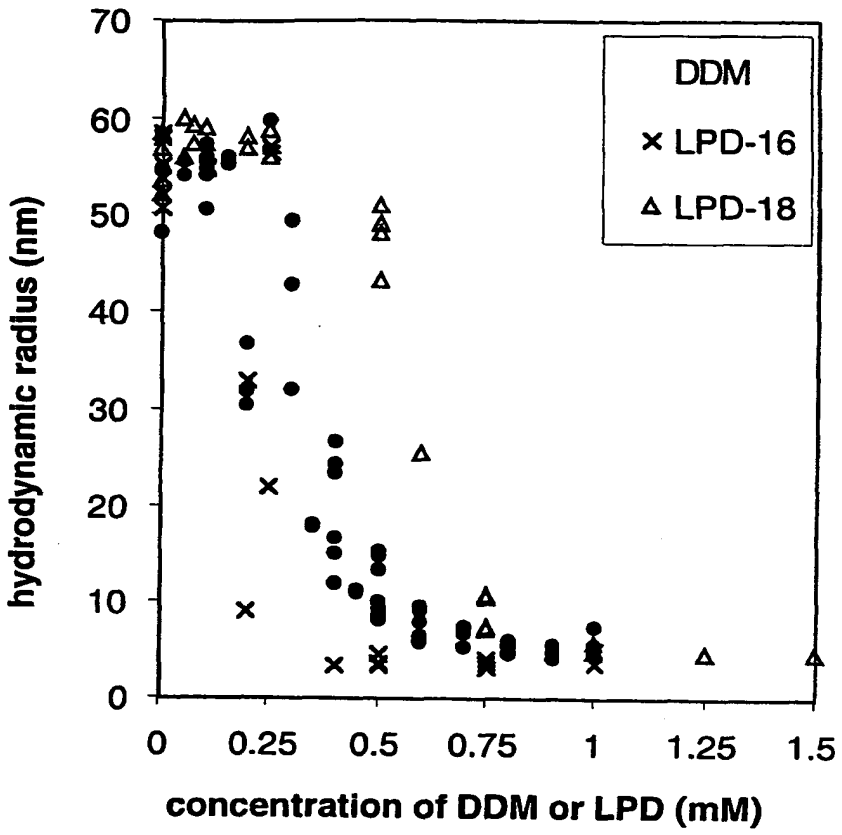
70
60
50
40
30
20
10
0
hydrodynamic radius (nm)
0
0.25
0.5
0.75
1
1.25
1.5
concentration of DDM or LPD (mM)
DDM
LPD-16
LPD-18

Figure 3.